Proteolytic and Chemical Modification of Colicin E3 Activity†

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ABSTRACT: Proteolyses of colicin E3 by both trypsin and subtilisin yield fragments of various molecular weights. On the basis of sodium dodecyl sulfate gel electrophoresis, tryptic cleavage yields peptides of molecular weight about 42 000 and 18 000, while the comparable pieces in a subtilisin digest have apparent weights of about 36 000 and 24 000. The digests lose almost all of their in vivo cell killing activity but the in vitro activity leading to ribosomal inactivation is augmented. Trypsin-treated colicin E3 shows a 20–30-fold increase in its ability to release the 52 nucleotide fragment from the 16S ribosomal ribonucleic acid (rRNA) and this activity is associated with the smaller fragment. Subtilisin-treated colicin E3 is only about two to threefold more active than the native protein in vitro, and the peptides obtained upon cleavage cannot be separated by gel filtration or polyacrylamide gel electrophoresis without sodium dodecyl sulfate. However, in the presence of 0.1% sodium dodecyl sulfate, subtilisin-treated E3 shows a 20–30-fold augmentation in in vitro activity which is again associated with the smaller fragment extracted from the sodium dodecyl sulfate gel. Amino terminal end-group studies showed that the two larger fragments and intact E3 have the same N-terminal residue, valine. These fragments presumably originate from the amino end of the native protein. The smaller tryptic fragment has an N-terminal alanine, while the smaller subtilisin piece has an N-terminal leucine. In addition, modification of a single carboxyl group in intact colicin E3 abolishes more than 90% of the in vivo activity with a simultaneous increase in in vitro activity. This carboxyl group is located in the larger fragments obtained in both trypsin and subtilisin cleavage. Binding of E3 to sensitive cells is drastically reduced or eliminated by this chemical modification and by both of the limited proteolytic cleavages.

Colicins are proteins produced by certain strains of *Escherichia coli* that can kill sensitive strains by a variety of different and specific modes of action (Nomura, 1963; Luria, 1964). Colicin E3 is characterized by its ability to inhibit protein synthesis by hydrolyzing one particular internucleotide bond near the 3′ end of the 16S rRNA (Samson et al., 1972). Identical cleavage products are obtained both in vivo when sensitive cells are treated with colicin E3 and in vitro when ribosomes are incubated directly with this colicin (Boon, 1972; Bowman, 1972). These findings suggest that the toxin may have to enter the cell, or at least penetrate through the plasma membrane, to contact the ribosomes. We previously reported that although Sephadex-bound E3 can bind to sensitive cells at their specific receptor sites, the cells are not killed. In contrast, with Sephadex-bound E1 both specific binding and killing are observed (Lau and Richards, 1976). The target for E1 is at the membrane level (Fields et al., 1969), while E3 appears to have to enter the cell (Boon, 1972; Bowman, 1972).

The present study began as a result of some observations on changes in properties of colicin E3 samples on storage. Freshly prepared E3 appeared as a single band on sodium dodecyl sulfate–polyacrylamide gels. However, after 1 to 2 months of storage at 4 °C in 0.01 M potassium phosphate buffer, pH 7, low-molecular-weight fragments began to appear. The aged samples showed a decrease in in vivo cell killing activity, but the in vitro ribosomal inactivation activity was increased. The absence of subunits in the native enzyme and the appearance of low-molecular-weight fragments upon aging suggested that proteolysis may have occurred perhaps as a result of protease contamination during purification. Stimulation of in vitro activity by trypsin treatment had been observed by Ohsumi et al. (1974) but further investigation into the products of the digestion has not been reported. A direct investigation of proteolytic cleavage was thus undertaken leading to the fragmentation patterns and activity changes reported below.

Experimental Procedures

Materials

The labeled compounds, [^{14}C]phenylalanine (405 mCi/mmol), sodium [^{3}H]borohydride (2.5 Ci/mmol), [^{14}C]glycine ethyl ester (5 mCi/mmol) were obtained from the New England Nuclear Corp. The following reagents were obtained from the suppliers indicated: 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide from Aldrich; *p*-chloromercuribenzoate, poly(U), ATP, GTP, glutathione, amino acids, phosphoenolpyruvic acid from Sigma; 1-ethyl-2-[3-(1-ethylnaphthol]-[1,2-*d*][thiazolin-2-ylidene)-2-methylpropenyl]naphthol[1,2-*d*]thiazolium bromide (stain all) from Kodak; dansyl chloride (10%, w/v, in acetone) and dansyl amino acids from Pierce; Cheng-chin polyamide TLC sheets from Gaillard-Schlesinger.

Trypsin (three times crystallized, ribonuclease free) was a gift from Dr. Joseph Fruton. Commercially supplied samples were: subtilisin from Novo, chymotrypsin from Worthington, soybean trypsin inhibitor from Sigma, and phosphoenolpyruvate kinase from Calbiochem.

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[1] Abbreviations used are: E3, colicin E3; E3-T, total tryptic digest of E3; E3-S, total subtilisin digest of E3; E3-T1, E3-T2, the large and small peptide fragments isolated from E3-T; E3-S1, E3-S2, the large and small peptide fragments isolated from E3-S; PCMB, *p*-chloromercuribenzoate; poly(U), poly(uridylic acid); ATP, GTP, adenosine and guanosine triphosphates; TLC, thin-layer chromatography; TM, 50 mM Tris-HCl, pH 7.5, 10 mM magnesium acetate, 5 mM ammonium chloride; EDTA, (ethylenedinitrilo)tetraacetic acid; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

The bacterial strains used were the same as those listed previously (Lau and Richards, 1976) and kindly supplied by Dr. Barbara Bachman from the *E. coli* Genetic Stock Center of Yale University: W3110 (col E3) was used for the preparation of E3. The test strains were wild type K12 (sensitive), A592 (tolerant), and A593 (resistant). Ribosomes for the in vitro assays were prepared from wild type K12.

Colicin E3 was prepared as previously described by Lau and Richards (1976) using the Konisky and Nomura (1967) modification of the Herschman and Helinsky (1967) procedure. E3 immunity factor was purified according to the method of Jakes (1973).

Tritium labeled E3 was prepared by methylation of free amino groups as described by Means and Feeney (1968). To each ml of a stock solution of colicin E3 (5 mg/ml) in 0.01 M potassium phosphate buffer, pH 9, was added 2.5 μl of 10 M formaldehyde followed by five consecutive additions of 5-μl aliquots of a solution of sodium borotritide, 0.8 M, 1.25 Ci/mmol. The reaction was stopped by the addition of acetone and the mixture was passed quickly through a 1 × 8 cm column of Sephadex G-25 to remove the nonprotein-bound counts. The void volume eluate was used directly in the cell-binding experiments.

For the preparation of ribosomes, K12 cells were harvested in log phase and an S30 extract was prepared as described by Webster et al. (1967). The extract was centrifuged in a Beckman Ti50 rotor for 5 h at 30 000 rpm at 4 °C. The upper half of the supernatant liquid was removed and used as the S100 preparation for the in vitro assay. The ribosomal pellet was suspended in TM buffer (50 mM Tris-HCl, pH 7.5, 10 mM magnesium acetate, 5 mM ammonium chloride), washed twice with 20 volumes of TM buffer and resuspended in TM buffer to give a concentration of 800 A_{260} units/ml.

Methods

Tests for Binding. Log-phase cells (sensitive, tolerant, or resistant) were mixed with different concentrations of ^{3}H-labeled E3 or its proteolytic digestion products. The cells were washed several times with growth medium to remove unbound labeled material and were then dissolved by heating at 100 °C for 5 min in 1% sodium dodecyl sulfate. The solutions were counted in a scintillation counter.

Assays for Colicin E3 Activity. For killing action on intact cells the spot and survival tests were used as previously described (Lau and Richards, 1976).

The in vitro activity was measured either by the inhibition of protein synthesis or by the direct estimation of cleavage of 16S rRNA. For the former, various concentrations of E3 or its digestion products in 0.01 M potassium phosphate buffer, pH 7, were incubated with 1.5–2.0 A_{260} units of purified ribosomes for 30 min at 37 °C. Excess immunity factor was added to terminate the reaction. The incorporation of [^{14}C]-phenylalanine into acid-insoluble counts was then tested by adding 50 μl of TM buffer containing 3 mM ATP, 0.2 mM GTP, 10 mM phosphoenolpyruvate, 2.5 mM glutathione, 20 μg of phosphoenolpyruvate kinase, all amino acids except phenylalanine at 0.1 M, 10 μl of S100, 3 μl of poly(U) (3 mg/ml), and 0.05 mM [^{14}C]phenylalanine. After further incubation for 20 min, the mixture was precipitated with 10 volumes of ice-cold Cl_3CCOOH (5%, w/v). The precipitate was filtered on Whatman 3MM paper and counted in a liquid scintillation counter. The maximum inhibition that was obtained in this assay even at high colicin concentration was about 80%.

For the second in vitro assay, E3 or its modified products were incubated with 1.5–2.0 A_{260} units of purified ribosomes. The reaction was stopped by adding sodium dodecyl sulfate to a concentration of 0.5%, EDTA to 1 mM, and the mixture was allowed to stand for 5 min at room temperature. The RNA was extracted by adding an equal volume of water-saturated phenol at 4 °C. The extract was precipitated by adding 2 volumes of ethanol and potassium citrate to a final concentration of 0.3 M. After 30 min at −20 °C, the precipitate was collected by centrifugation for 20 min at 25 000g. The precipitate was dissolved in 40% sucrose containing 2 mM EDTA and 10 mM Tris, pH 8, and this solution was fractionated by electrophoresis on a 10% gel. The results could be roughly quantitated by scanning the stained gels on a Joyce-Loebl densitometer and comparing the integrated intensity of the E3 RNA fragment with that of the 5S band in the same sample. Within the appropriate range the amount of the 52 nucleotide fragment produced was proportional to the amount of colicin used.

Proteolytic Digestions. A stock solution containing approximately 5 mg of colicin E3/ml was prepared in 0.01 M potassium phosphate buffer, pH 7. Stock solutions of trypsin, subtilisin, and chymotrypsin were prepared in the same buffer at an enzyme concentration of 1 mg/ml in each case. Unless otherwise indicated the E3/enzyme ratio used for the digestions was 50:1 for trypsin and chymotrypsin and 100:1 for subtilisin. The digestions were all carried out at 37 °C. The incubation time was normally 20 min. The reactions were stopped by the addition of soybean trypsin inhibitor for trypsin, and *p*-chloromercuribenzoate for subtilisin.

Polyacrylamide Gel Electrophoresis. Several different systems were used. Analytical sodium dodecyl sulfate disc gels (10 and 15%) were prepared and run following Fairbanks et al. (1971). Preparative gels were run in the same manner except that a dansylated sample of the same material was run in parallel. The positions of the bands in the preparative gels were thus quickly established. The bands were immediately cut out and extracted in the gel buffer containing 0.1% sodium dodecyl sulfate after completion of the run.

The Laemmli (1970) gel system was used with mini-slab gels, 6 × 4 × 0.1 cm. Normally, six samples were run for 2 h in each gel at 7 mA/gel. The protein gels were stained with 1% Coomassie blue.

Slab gels without sodium dodecyl sulfate were run according to Jakes (1973) with the addition of a 5% stacking gel on top of the running portion.

Slab gels for separating RNA samples were prepared and run as described by Peacock and Dingman (1968) with the addition of a 3% stacking gel on top of the 10% running gel. The RNA was stained using stain all as described by Darlberg et al. (1969).

Dansylation and N-Terminal Amino Acids. The proteins were dansylated in the presence of sodium dodecyl sulfate according to the method of Weiner et al. (1972). The dansylated proteins were either run directly as markers or hydrolyzed in 6 M HCl for 8 h at 104 °C for end-group analysis. The gel bands whose N-terminal residues were to be identified were extracted as described above and then also hydrolyzed in 6 M HCl for 8 h at 104 °C.

The N-terminal amino acids were identified on 5 × 5 cm polyamide-layer sheets by comparing with standards run simultaneously on the opposite side of the same sheet. The chromatograms were developed with the solvent systems described by Hartley (1970).

Carboxyl Group Modification. Amidation of carboxyl groups was carried out according to the method of Hoare and Koshland (1967). A solution of E3 (5 mg/ml) was adjusted

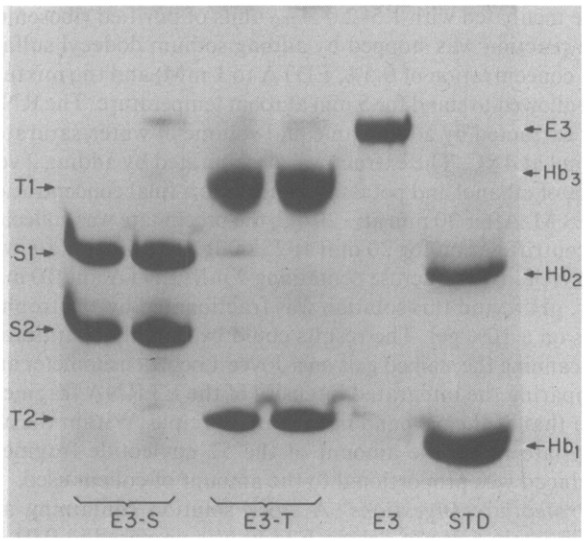


FIGURE 1: Sodium dodecyl sulfate gel separation of E3 fragments in the Laemmli (1970) system. The fragments and standards are identified by the ordinate labels. The undigested E3 sample contained 3 μg of protein. For the digests, 9 μg of total protein was applied to the gel for each sample. For the trypsin pair, E3-T, the weight ratio of E3 to enzyme was 50:1. The digestion time for the right sample was 10 min, the left 20 min. For the subtilisin pair, E3-S, the weight ratio was 100:1 with digestion times of right sample 10 min, and left sample 20 min. The standards shown on this particular gel consist of the components of a lightly cross-linked sample of hemoglobin showing monomer, dimer, and a faint trace of trimer.

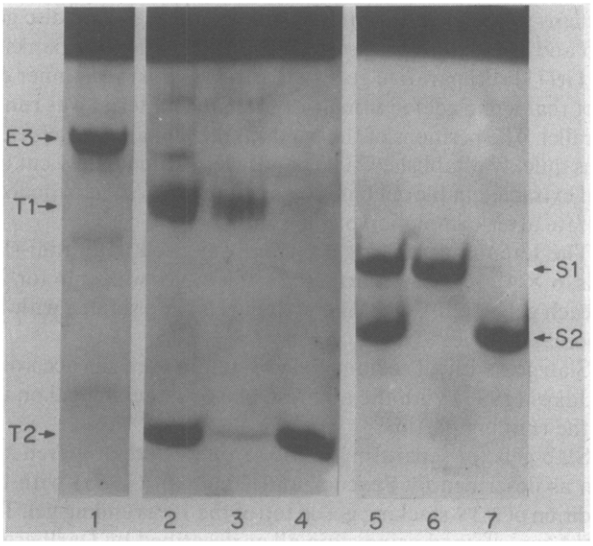


FIGURE 2: Sodium dodecyl sulfate gel separation of E3 fragments in the Laemmli (1970) system. Sample 1, 3 μg of E3 undigested. Sample 2, 9 μg of a tryptic digest of E3, weight ratio of E3 to enzyme 50:1, digestion time 20 min. Samples 3 and 4, peaks 1 and 2 from Bio-Gel separation of digest (see Figure 4). Sample 5, 9 μg of a subtilisin digest of E3, weight ratio 100:1, digestion time 20 min. Samples 6 and 7, reruns of E3-S1 and E3-S2 bands eluted from a preparative sodium dodecyl sulfate gel separation of this digest, 4 μg of protein in each sample.

to pH 4.75 with dilute HCl and [^{14}C]glycine ethyl ester was added to a final concentration of 0.1 M. The water-soluble reagent 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide was then added to a final concentration of 0.05 M to initiate the reaction. The reaction mixture was left at room temperature for 30 min and stopped by addition of 1 ml of 1 M acetate buffer, pH 4.75. The mixture was dialyzed against 0.01 M potassium phosphate buffer (pH 7) for 36 h. From the known specific activity of the reagent, the extent of incorporation of

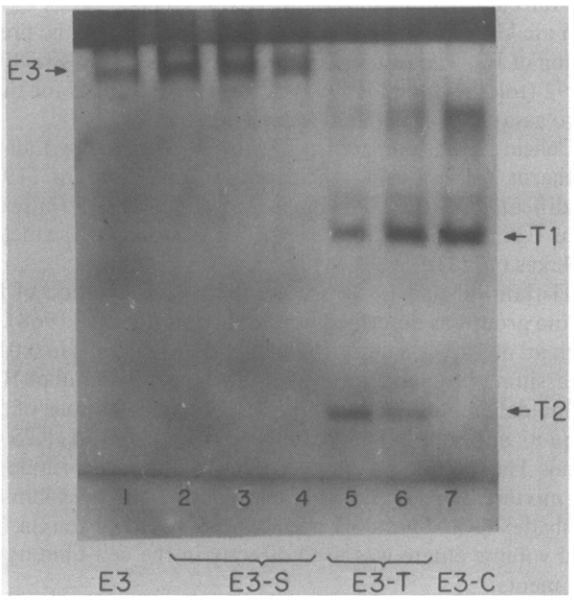


FIGURE 3: Polyacrylamide gel runs (10%, no sodium dodecyl sulfate) of E3 and tryptic and subtilisin digests. The times of the subtilisin digestions were 10, 20, and 35 min for samples 2, 3, and 4, respectively, at a weight ratio of E3:S of 100:1. For the tryptic digest, samples 5 and 6, the times were 20 and 40 min at a ratio of 50:1. For chymotrypsin, sample 7, the digestion time was 20 min and the ratio 50:1.

the isotope corresponded to about 1.1 mol of glycine ethyl ester/mol of E3.

The ^{3}H-labeled E3 was also modified in a similar manner but with nonradioactive glycine ethyl ester to yield a product used in the binding assay. The modified E3 was either used directly in activity assays or was treated with either trypsin or subtilisin to yield digestion products comparable to those obtained from unmodified E3.

Results

Digestion and Separation of Fragments. Samples of freshly prepared E3 showed one principal band on gel electrophoresis at a position corresponding to an apparent molecular weight of about 60 000, Figures 1, 2, and 3. This was also true in the Fairbanks et al. (1971) system (results not shown). With heavily loaded gels, faint bands appeared in other positions but no amount of immunity factor comparable to the principal band was seen in any of the preparations.

Limited proteolytic digestion of E3 with either trypsin or subtilisin produced two clearly defined peptide products separable on sodium dodecyl sulfate gels from E3 and from each other, Figure 1. Approximate molecular weights for these fragments were estimated from gels calibrated with bovine serum albumin, ovalbumin, and lightly cross-linked hemoglobin, Table I. Considering the probable error of 5–10% for molecular weight determinations by gel electrophoresis, Weber and Osborn (1969), the two gel systems yield essentially identical values. The agreement between the fragment sums and the starting molecular weight is obviously fortuitous. It is clear that the fragments represent most of the original molecule, but the loss of small peptides aggregating even as much as 5000 in molecular weight cannot be ruled out on the basis of presently available data. In the early stages of digestion no dye-staining bands other than the main fragments are seen

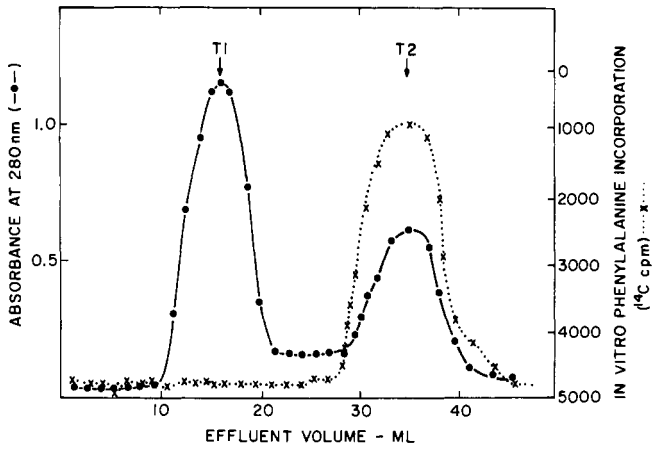


FIGURE 4: Separation of the tryptic fragments of colicin E3 on a column of Bio-Gel P60. The column size was 0.65 cm^2 × 45 cm with a void volume of 9.1 ml. The elution buffer was 0.01 M potassium phosphate, pH 7.0. The absorbance at 280 nm of the column effluent is shown on the left-hand ordinate (- ● -). In vitro biological activity was measured by inhibition of poly(U)-directed phenylalanine incorporation into acid-insoluble material. The actual measured counts ([^{14}C]phenylalanine) in the assay are shown on the right-hand ordinate (··· X ···).

on the gels. However, the limited proteolysis is only relative. Higher ratios of enzyme to substrate or longer digestion times produced additional cleavage. By the time the E3 band had disappeared, multiple faint fragments could be seen and exact stoichiometry is thus uncertain. The sodium dodecyl sulfate gel patterns are identical in the presence or absence of reducing agent in the buffer. Thus, disulfide bonds do not appear to play a role in the association of these fragments.

Cleavage with chymotrypsin produced only one moderately well-defined fragment with multiple smeared bands elsewhere on the gel (see the last sample in Figure 3). Since the digest rapidly lost all of the in vivo and in vitro activity of the starting E3 material, no further studies were carried out with this enzyme.

The tryptic fragments were separated preparatively on a gel-filtration column composed of Bio-Gel P60. A typical pattern is shown in Figure 4. Samples from the two peaks run on sodium dodecyl sulfate analytical gels and compared with the original digest are shown in Figure 2. The separation is quite clean and unambiguous. The subtilisin fragments, however, could not be separated in the absence of a denaturing agent. In the gel pattern shown in Figure 3 the digested sample moves as a single band at the same position as undigested E3. On a Bio-Gel column, E3 and the digest ran together in precisely overlapping peaks. The subtilisin fragments had to be separated electrophoretically on preparative sodium dodecyl sulfate–polyacrylamide gels. The purity of the resulting fragments from extracts of pooled preparative runs is indicated in Figure 2.

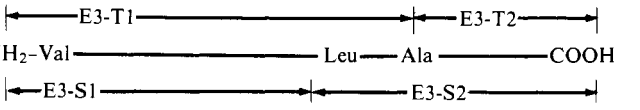
End-Group Determinations and Chemical Modification. Chromatograms of the products of the dansyl end-group procedure applied to E3 and its various proteolytic fragments are shown in Figure 5. The amino terminal residue of the intact colicin appears to be valine. The predominant product in each case from fragments E3-T1 and E3-S1 is also valine. The amino end group on E3-T2 is alanine, while that for E3-S2 is leucine. In the absence of more complete sequence data or information on the presence or absence of more than a single peptide bond cleavage, no definitive conclusions can be drawn. However, the simplest explanation of the present data would be:

TABLE I: Approximate Molecular Weights of E3 Fragments from Sodium Dodecyl Sulfate Gel Electrophoresis.

Sample	Laemmli System	Fairbanks System
E3	$60 \pm 1 \times 10^3$	$60 \pm 1 \times 10^3$
E3-T1	$42 \pm 2 \times 10^3$	$44 \pm 2 \times 10^3$
E3-T2	$18 \pm 1 \times 10^3$	$16 \pm 1 \times 10^3$
E3-S1	$36 \pm 1 \times 10^3$	$37 \pm 2 \times 10^3$
E3-S2	$24 \pm 1 \times 10^3$	$23 \pm 1 \times 10^3$

TABLE II: Relative Activities of Colicin E3 Derivatives.

Preparation	Relative Sp Act. In Vitro	Relative Sp Act. In Vivo
Intact E3	1	1
Total tryptic digest, E3-T	20–30	<0.05
Large fragment, E3-T1	<0.3	<0.01
Small fragment, E3-T2	20–30	<0.01
Total subtilisin digest, E3-S	2–3	<0.05
Total subtilisin digest in 0.1% sodium dodecyl sulfate	20–30	<0.01
Large fragment in 0.1% sodium dodecyl sulfate	<0.2	<0.01
Small fragment in 0.1% sodium dodecyl sulfate	20–30	<0.01
Carboxyl modified E3	2–3	<0.01



The indicated lengths of the peptides are in approximate proportion to their apparent molecular weights.

Gentle treatment of E3 with a water-soluble carbodiimide and labeled glycine ethyl ester produced a derivative in which the extent of label incorporation indicated the modification of a single carboxyl group (see Materials and Methods). The total protein sample lost about 90% of its in vivo killing activity, while its in vitro activity was increased (Table II). The loss of in vivo activity was caused by, or accompanied by, failure of the derivative to bind to sensitive cells. Little or no direct binding was observed nor did the derivative protect sensitive cells against attack by native colicin. When this carboxyl-modified derivative was treated with trypsin or subtilisin, the characteristic fragmentation patterns were observed. The gels were sliced and counted to locate the ^{14}C label of the glycyl ester modifying group. The isotope was located in the bands designed E3-T1 and E3-S1, Figure 6.

The modification of amino groups by the formaldehyde-borohydride procedure of Means and Feeney (1968) produced a heavily tritiated product but did not appear to affect either the in vivo or in vitro activity of E3. A rough estimation of the actual extent of modification was made by monitoring the disappearance of free primary amino groups by the trinitrobenzenesulfonate procedure. The product had lost about half of the amino groups present in the original E3 sample. The proteolytic cleavages and the carboxyl group modification reaction proceeded exactly as with the native colicin. This tritium-labeled colicin was used in the binding studies of E3 and its derivatives to sensitive cells (Figure 9).

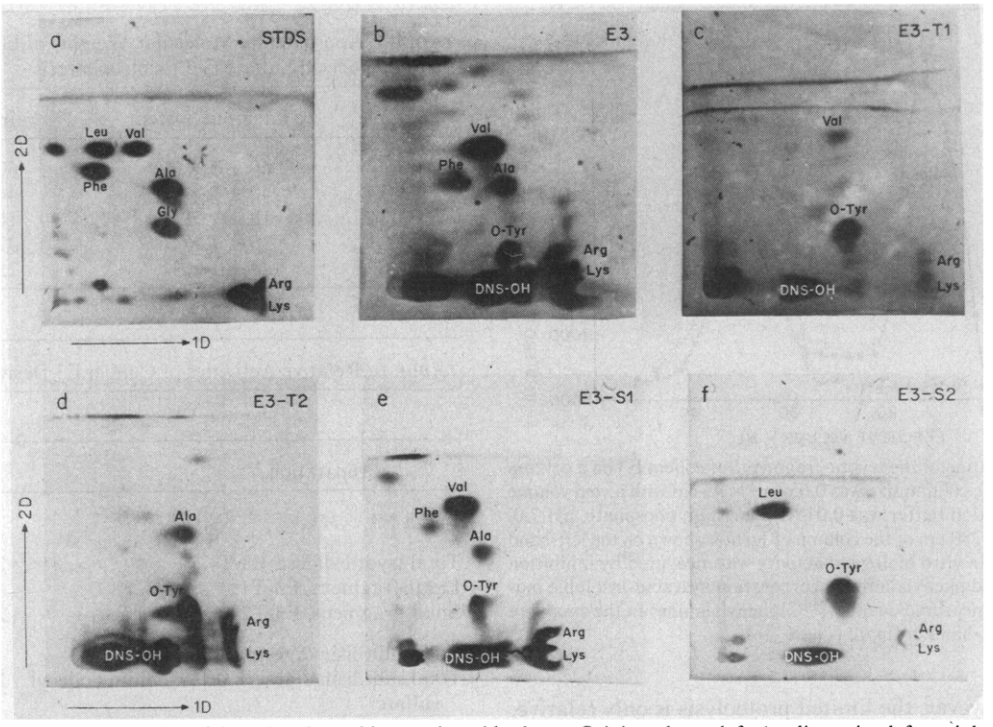


FIGURE 5: Two-dimensional separation of dansyl amino acids on polyamide sheets. Origin at lower left, 1st dimension left to right 1.5% formic acid, 2nd dimension bottom to top chromatography in benzene–glacial acetic acid in a ratio of 9:1. Each panel is an autopositive print of the negative taken of the fluorescence of the sheets after chromatographic development. Panel a shows the combined reference standards. Panels b–f show the end-group estimates from hydrolysates of dansylated samples of E3 and its various proteolytic fragments as indicated. Varying amounts of Lys, O-Tyr, and dansylic acid (DNS-OH) are seen in each hydrolysis sample. The gel shown in b for intact E3 was very heavily loaded. The principal contaminants appear to be phenylalanine and alanine. The principal end group is clear in each case, although the small amounts of phenylalanine and alanine in the E3 sample are also seen in E3-S1 and just barely visible in the E3-T1 gel shown.

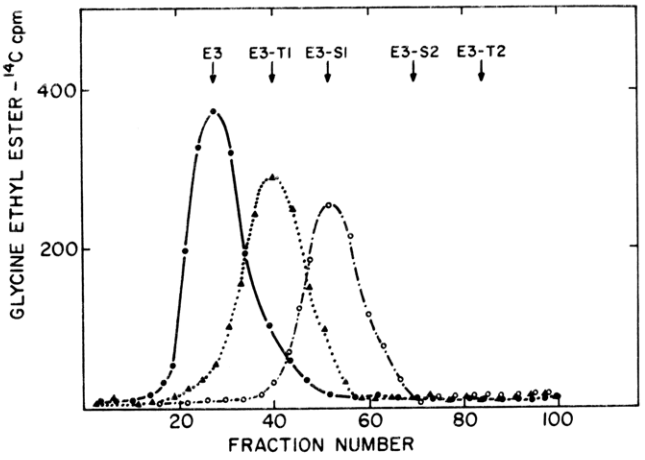


FIGURE 6: Distribution of the ^{14}C label of glycyl ester groups in sodium dodecyl sulfate gel runs of colicin E3 and its tryptic and subtilisin digests. The origin of the gels is at the left at fraction 1. The samples, comparable to those shown in Figure 1 were: colicin E3 (— ● —); total tryptic digests (· · · ▲ · · ·); total subtilisin digest (- - - ○ - - -). The positions of the Coomassie blue staining bands in the different runs are shown and identified by the labeled arrows.

Properties of Colicin E3 Digests and Isolated Fragments. A tryptic digest of E3 that showed little or no intact colicin remaining lost essentially all of its in vivo killing activity. However, the in vitro activity on isolated ribosomes was enhanced. The inhibition of labeled amino acid incorporation into acid-insoluble counts is shown in Figure 7. Under the conditions used, incubation of 10 μg of E3 with a fixed amount of ribosomes gave 50% inhibition in about 15 min. Slightly faster inhibition was obtained with 0.5 μg of the total tryptic digest implying an increase in activity of more than 20-fold. In Figure 4, showing the column separation of the two tryptic fragments, it can be seen that all of the in vitro activity is associated with the smaller fragment E3-T2.

It was possible that a change in specificity of the nucleolytic activity of E3 had occurred on proteolysis or alternatively that the trypsin reagent had nuclease contamination as occurs frequently with commercial samples. In either case, nonspecific nucleolytic attack on the protein-synthesizing system might have been the explanation for the apparent increase in in vitro activity. This point was checked by examining the total RNA content of ribosomes exposed to E3 or one of its modified products. The specific 52 nucleotide piece is easily recognized by its position in the RNA gel pattern. The results of such tests are shown in Figure 8, upper panel. Neither the control nor E3-T1 showed any of the specific fragment, while E3, E3-T (the total digest), and E3-T2 (small fragment) yielded the specific RNA piece, with the latter sample producing the largest amount. Cleavage of the RNA with less specific nucleases led to a smeared uninterpretable pattern.

Subtilisin-treated E3 also showed an increase in in vitro ribosomal-inactivation activity. Assay for the in vitro activity was done primarily by quantitating the intensity of the specific nucleotide on RNA gels. The in vitro [^{14}C]phenylalanine-incorporation assay did not serve as a good test for the activity because the inhibitor (PCMB) used to stop the subtilisin action seemed to interfere with the rate of amino acid incorporation, as did the presence of sodium dodecyl sulfate in some of the subtilisin–E3 samples. Unlike E3-T, subtilisin-treated E3, E3-S, only showed a two to threefold increase in the in vitro activity (Table II) despite the fact that more than 95% of the protein had been cleaved into two fragments, as shown by gel electrophoresis (Figure 1). However, in the presence of 0.1% sodium dodecyl sulfate, E3-S showed the same augmentation

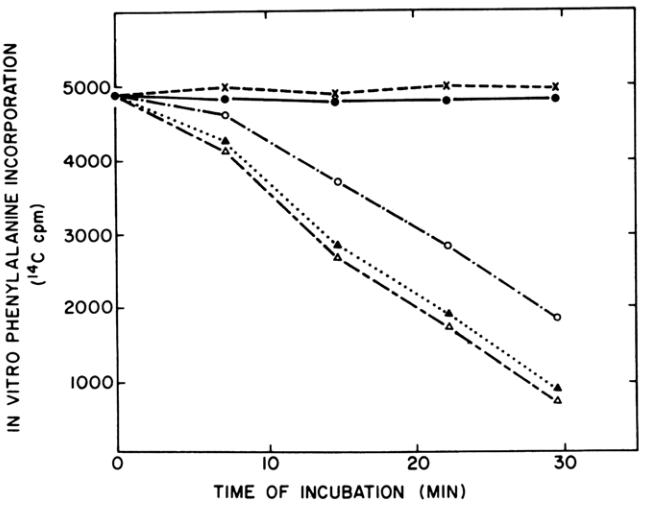


FIGURE 7: Inactivation of ribosomes by colicin E3 and a tryptic digest of this protein. Aliquots of a solution of purified ribosomes in an amount equivalent to 2.5 A_{260} units were incubated with different amounts of E3 or its tryptic digest. At various times samples were removed and tested for their ability to cause the poly(U)-directed incorporation of [^{14}C]phenylalanine into acid-insoluble counts. The measured counts in the filtered precipitate are shown on the ordinate as a function of the incubation times of the protein sample with the ribosomes. (X - - - X) control samples, no colicin added; (● – ●) E3, 1 μg; (▲ ··· ▲) E3, 10 μg; (O - · - · O) E3-T, 0.05 μg; (Δ - - - Δ) E3-T, 0.5 μg.

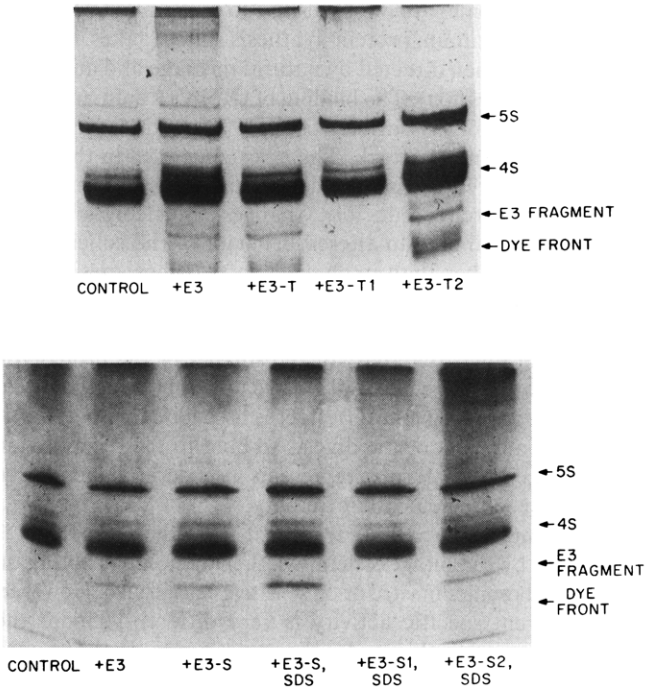


FIGURE 8: Polyacrylamide gels of RNA extracted from ribosomes exposed to colicin E3 or its proteolytic digestion products. The large RNA molecules do not enter the gel. The positions of the 5S, 4S, and specific 52 nucleotide E3 fragment are indicated. The upper panel shows the effect of E3 (5 μg), E3-T, the total tryptic digest (0.5 μg), and the fragments E3-T1 (0.4 μg) and E3-T2 (0.2 μg) mixed in each case with an amount of ribosomes corresponding to 2.5 A_{260} units. The lower panel shows a comparable series for subtilisin: E3 (5 μg), E3-S (5 μg), E3-S (0.5 μg) in 0.1% sodium dodecyl sulfate, E3-S1 (0.4 μg) in 0.1% sodium dodecyl sulfate, and E3-S2 (0.2 μg) in 0.1% sodium dodecyl sulfate.

in in vitro activity as E3-T (Figure 8, lower panel). In the same figure it can be seen that E3-S2 is responsible for the nucleolytic activity, while E3-S1 is inactive.

Using tritiated E3 and its digests, a direct estimate was made of binding of these materials to sensitive cells. The results are shown in Figure 9. All of the added E3 is bound up to a saturation level that in this particular experiment corresponded to about 1000 cpm. Only very slight additional binding is seen at higher levels of E3 beyond this point. The digests prepared with either trypsin or subtilisin show less than 5% as many counts bound at the equivalence point. The small amount of observed binding could be due either to a trace of undigested E3 or to the nonspecific binding seen at higher levels with the native colicin. No binding was observed with the isolated tryptic fragments nor did they inhibit binding of intact colicin. There is no evidence so far that any of the proteolytic fragments or the carboxyl-modified derivative can bind to the receptors on sensitive cells.

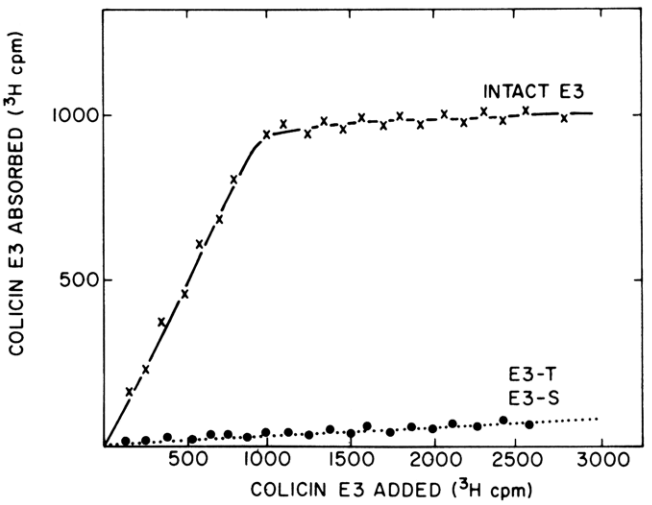


FIGURE 9: Binding of ^{3}H-labeled colicin E3 and the proteolytic digests to sensitive cells. The labeled sample containing the number of counts shown on the abscissa was mixed with 10^{8} cells and incubated at 37 °C for 30 min. The washed cells were dissolved and counted to yield the ordinate values. The data for the tryptic (E3-T) and subtilisin (E3-S) digests were indistinguishable and are shown as a single line.

Discussion

Colicin E3 appears to have a domain-type structure with at least two unequal self-structured regions connected covalently by the peptide chain but otherwise loosely associated. There are specific peptide bonds, or at most small regions, on the surface that are highly susceptible to proteolytic attack by either trypsin or subtilisin. With tryptic cleavage, the residual noncovalent interactions between the two domains are not strong enough to prevent easy separation of the two pieces. The subtilisin cleavage point is some 50–70 residues closer to the N-terminus of the original chain. The interactions between this N-terminal part of the E3-S2 and the E3-S1 fragments are sufficiently large that the two pieces now do not dissociate in the absence of a denaturing agent such as sodium dodecyl sulfate. However, after separation, the properties of the E3-S2 fragment are indistinguishable from those E3-T2. Thus, the structure of the small domain does not appear to be affected by the extra tail present in the E3-S2 version.

There are apparent stringent conformational requirements for the inactivation of ribosomes by colicin E3 (Boon, 1972; Bowman, 1972; Darlberg et al., 1973). Ribosomes must be in the 70S form to be susceptible to E3. Both purified 30S subunits and isolated 16S rRNA are resistant to the specific nucleolytic attack of the toxin (Boon, 1972). The extent and rate of inactivation, however, are strongly influenced by other

factors. Ribosomes are most sensitive to inactivation when they are in their optimum protein-synthesis states (Jakes and Zinder, 1974) (when directed by natural message and at 10 mM Mg^{2+} concentration). The binding of tRNA (Kaufmann et al., 1973) or antibiotics (Darlberg et al., 1973) tends to protect the ribosomes from E3 attack. The ribosomes used in this work were all prepared and used under a standard set of conditions The results were reproducible. Changes in nucleolytic activity were thus attributed to altered behavior of the colicin or its derivatives rather than to changes in the ribosomes used as substrates.

The two principal observations in this study are the dramatic increase in in vitro activity of the smaller of the proteolytic fragments and the equally dramatic loss of in vivo activity of the total digests. The latter effect is associated with a loss of ability of any part of the digests to bind to the receptors on sensitive cells. The former represents a substantial increase in a highly specific nucleolytic activity.

The same 52 nucleotide fragment from the 16S rRNA is produced by the intact colicin whether the toxin is acting on isolated ribosomes in vitro or on sensitive cells in vivo. However, the apparent specific activity is very different. Under the conditions used in this study, the particle ratio in the in vitro assay is 5 colicin E3:1 ribosome for complete 16S RNA cleavage in a 30-min incubation. Thus, it is not certain that the hydrolysis of the nucleic acid is even catalytic. In this work we found that about 1000 colicin molecules were needed to kill one sensitive cell. When ribosomes from E3 treated cells were isolated and the rRNA was extracted, more than 90% appeared to be specifically cleaved in agreement with Samson et al. (1972). Assuming at least 10 000–15 000 ribosomes/cell (Lehninger, 1972) and assuming that all of the adsorbed colicin molecules enter the cell, each colicin molecule would have to inactivate ten or so ribosomes during a comparable 30-min incubation. This would correspond to an increase in specific activity of 50 between the in vitro and in vivo conditions. The number would be higher if only a fraction of the toxin molecules actually entered the cell.

The increase in activity might be caused by: (1) dissociation of immunity factor as proposed by Jakes (1974), (2) interaction with a specific factor in the cell interior as suggested by the observations of Oshumi et al. (1974), or (3) cleavage by a trypsin-like protease inside the cell. The E3 prepared in this work appeared to have little or no immunity factor associated with it on the basis of gel patterns. If "factor" activation or proteolysis were involved it would have to occur in the cell interior or perhaps in the periplasmic space. The previous study (Lau and Richards, 1976) showed that Sephadex-immobilized E3 bound to sensitive cells but did not kill them, implying that all, or at least part, of the colicin molecule had to get physically through the inner membrane for killing action to be observed. A subtilisin-like cleavage in the cell interior would not result in sufficient activation, since the two chains would still be tightly associated in a relatively inactive complex. The nature of the structural change leading to activation that is produced on actual separation of the small and large fragments is, of course, unknown at this time.

The binding of E3 to its receptors on sensitive or tolerant cells is a very specific interaction. The modification of what is probably a single unique carboxyl group on E3 is enough to destroy the receptor binding. The cleavage of a single peptide bond, or at most a very few, by either trypsin or subtilisin at two quite different loci in the primary sequence is also enough to eliminate binding even before separation of the two chains so produced. The unique carboxyl group is located on the larger fragment and points to the importance of this domain of the E3 structure in the binding reaction. However, the involvement of both the small and large fragments is not ruled out. That some critical structural change is produced simply by the bond cleavage is shown by the behavior of the subtilisin digest where the two chains are still tightly coupled yet the binding ability is markedly lowered if not destroyed. Sabet and Schnaitman (1972) have reported that mild proteolysis of the receptor protein also eliminates the binding reaction.

There is at least a partial similarity of the colicin-E3 system with those bacterial toxins that are pathogenic to eukaryotic cells, such as diphtheria (Collier, 1975), tetanus (Craven and Dawson, 1973), staphylococci (Spero et al., 1975), and cholera (Cuatrecases et al., 1973), and even plant seed toxins such as ricin (Olsnes et al., 1974) and abrin (Olsnes et al., 1973). It is becoming clear that attachment to receptors on sensitive cells and the toxic action are separate functions, and that these functions are located on different peptide chains. The toxic action is not manifested until the chains are separated. In some cases the chains are linked by disulfide groups and thus reduction is a prerequisite for separation (Collier, 1975; Olsnes et al., 1973, 1974; Spero et al., 1975; Cuatrecases et al., 1973). In the case of diphtheria toxin, proteolytic cleavage is also required (Collier, 1975). However, the analogy is certainly not complete. In each of the above cases, the binding activity is retained on the nontoxic chain and this binding is sufficiently strong that the single chain can compete effectively for binding sites with the native toxin. The target eukaryotic cells, unlike the prokaryotic cells affected by colicins, do not have a cell wall and the toxins bind directly to receptors in the plasma membrane. In the prokaryotic cells, the receptors are largely, if not solely, located in the outer membrane layer. For the eukaryotic cells, endocytosis is a possible mechanism for entry of the toxin into the cell interior. This process cannot account for the transport problem in the prokaryotic system. In either system the mechanism by which these large toxin molecules gain entry to the cell interior clearly involves more than the receptor proteins and remains a general unsolved problem.

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Assignment of a Ligand in Stellacyanin by a Pulsed Electron Paramagnetic Resonance Method†

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ABSTRACT: The electron spin echo decay envelope for the blue copper protein, stellacyanin, and for a number of other Cu(II) complexes has been studied. Particular attention was given to the form of the "nuclear modulation" patterns, which show the effects of coupling between the electron spin and the neighboring nuclei. The envelopes for the hydrated cupric complex and for copper(II) glycylglycine were essentially the same and indicative of the coupling to protons. The peptide complex contains nitrogen nuclei coupled directly to Cu(II), but the coupling constant is so large for these nuclei that a modulation pattern ascribable to ^{14}N is not seen. For copper(II) bovine serum albumin, on the other hand, a contribution due to the coupling of the remote nitrogen belonging to a histidyl imidazole ligand was observed. The modulation pattern for this complex and for stellacyanin closely resembled one another, strongly suggesting that an imidazole is ligated to the copper in this blue protein.

Two types of mononuclear copper sites have been identified in copper proteins. Of these, the widest attention has been given to the dark blue, type I site, as is found in azurin (Suzuki and Iwasaki, 1962; Sutherland and Wilkinson, 1963; Gould and Mason, 1967; Brill et al., 1968), stellacyanin (Omura, 1961; Peisach et al., 1967), plastocyanin (Katoh et al., 1962; Blumberg and Peisach, 1966), umecyanin (Stigbrand et al., 1971), and a variety of copper-containing oxidases (Malmström and Vänngård, 1960; Blumberg et al., 1963, 1964, Poillon and Dawson, 1963; Nakamura and Ogura, 1966; Malkin et al., 1968a,b; Malmström et al., 1968; Nakamura et al., 1968; Andréasson and Vänngård, 1970). This is in part because of the unusual optical and magnetic properties associated with them. They are generally characterized by an extraordinarily intense optical absorption near 600 nm and a small value of the magnetic hyperfine coupling constant $A_\parallel$. Simple copper peptide complexes and copper in type II sites are, on the other hand, characterized by values of $A_\parallel$ several times larger and also are less intensely colored (Malkin and Malmström, 1970).

A comparison of the EPR[1] data for copper proteins and model compounds suggests that the metal ligands for type II sites in copper-containing oxidases are oxygen and/or nitrogen, sulfur being ruled out (Peisach and Blumberg, 1975). EPR studies on type I sites have not, however, provided any definite information regarding ligands and most of the evidence has been obtained by chemical means. One such study performed with parsley plastocyanin, a blue copper protein containing a single cysteinyl residue, strongly implicates cysteinyl sulfur as a ligand (Finazzi-Agró et al., 1970). Removal of the copper activates the cysteinyl sulfur for binding to *p*-mercuribenzoate. While in this mercurial-bound form, the protein cannot be reconstituted to the holoprotein with copper. Removal of the

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[1] Abbreviations used are: EPR, electron paramagnetic resonance; ENDOR, external nuclear double resonance; Cu(II)(imid)$_4$, copper tetraimidazole; Cu(II)(guan)$_4$, copper tetraguanidine.